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THE MYXOMYCETES

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CONTENTS

1. Classification

There is some confusion as to the limits of the class Myxomycetes. There are four orders within this class:[1]

1) The Myxomycetes - the true (acellular, noncellular, syncitial, plasmoidal) slime molds which will be the topic of this paper.

2) The Acrasiales. These are the cellular slime molds which resemble Myxomycetes in the production of myxamoebae, but do not produce true swarm cells or true plasmodia, and the aggregated amoebae can be easily shaken apart in water. The Acrasiales are the slime molds which are providing biologists with very interesting material for the study of differentiation.

3) The Plasmodiophorales. These include several genera, all parasitic on plants or fungi. They are similar to Myxomycetes but they lack a secreted sporangial wall and capillitial threads, they form zoosporangia and they have a distinctive type of nuclear division.

4) The Labyrinthulales. The taxonomic position of these organisms is uncertain. The assimilative stage consists of spindle or oval shaped cells gliding on or within slime tracks or tubes. The slime tubes are joined to form a network (the filoplasmodium) which is why these organisms are often known as the net slime molds. The filoplasmodium is unrelated to the plasmodium of the Myxomycetes.

2.   Life Cycle of the Myxomycetes

The spores germinate releasing from one to four uninucle-ate, haploid protoplasts. In water these develop flagella and are then called swarm cells. Where free water is lacking they become amoeboid. The amoeboid-phase organisms are called myxoamoebae. The myxamoebae can, if they reach free water, develop flagella and become swarm cells. Conversely, the swarm cells can resorb their flagella to become myxamoebae. If the food supply is insufficient or when other adverse conditions prevail, both phases can encyst, the swarm cells becoming myxamoebae before encystment. Both the swarm cells and the myxamoebae divide by binary fission, the swarm cells first resorbing their flagella. The swarming phase may last from several hours to many days.

When a certain critical concentration of cells is reached, compatible myxamoebae or swarm cells behave as gametes and copulate in pairs. It is not known whether copulation of myxamoebae as against swarm cells is a characteristic of individual species. Cell fusion is followed by nuclear fusion and diploid zygotes are formed. Heterothallism has now been adequately demonstrated, but as yet not widely observed.

The zygotes feed and grow. The nucleus divides but the cytoplasm does not, transforming the zygote into a plasmoidium. Nuclear division, is synchronised within a single plasmodium. Plasmodia fuse with one another, and with zygotes, enlarging

by accretion as well as by growth. This phenomenon provides an interesting system for the study of genetics (whether two genetically different plasmodia can fuse, and if so which nuclear type becomes dominant and why) and the synchrony of nuclear division.

When the conditions are right the plasmodium develops into a fruiting body (sporophore), the morphology of which is species specific, accompanied by cleavage of the protoplasm into uninucleate portions. These become enveloped by walls and mature into spores. The network of thread-like structures within the sporophore, the capillatum, usually develops at this point. Meiosis either occurs in the sporophore during spore cleavage[2,3] or in the spore after its formation[4]; there is some controversy over this point. It may be that meiosis does not occur at the same point in the life cycle in different species, or even the same isolate of the same species.

There is a genus of Myxomycetes whose life cycle differs from that described enough for it to be put into its own subclass; this is the genus Ceratiomyxa. The main difference is that it is exosporous; its spores are born on individual stalks. On germination each spore gives rise to 8 swarm cells. Its plasmodium is more conspicuous than those of the other (endosporous) Myxomycetes.

The life-cycle of the endosporous Myxomycetes can be summarized diagrammatically:
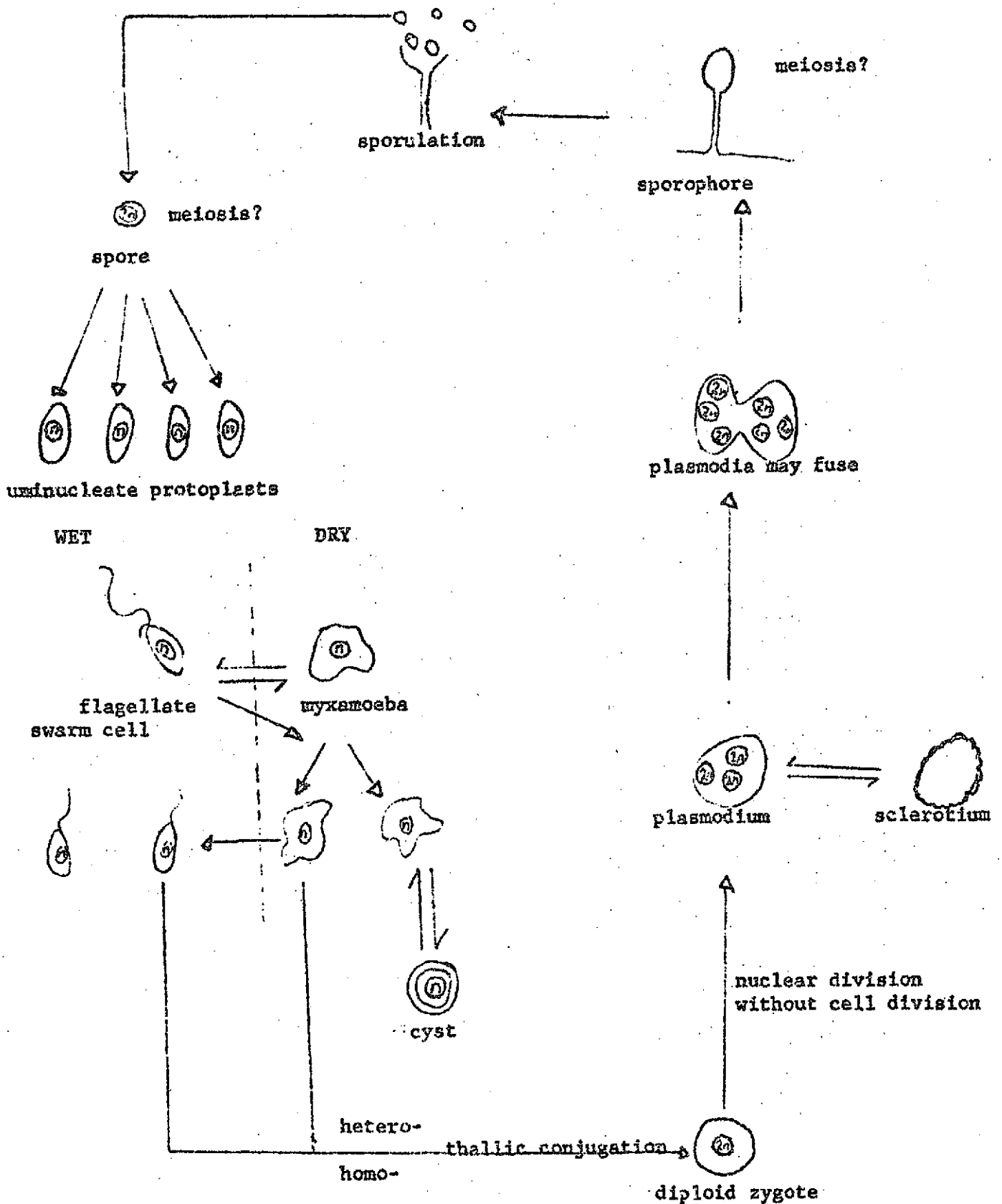
Diagrammatic representation of the life cycle of the Myxomycetes

(not to scale)

3. <u>Morphology and Physiology</u>

<u>Spores</u>. The spores are usually spherical. They show a wide range of colors, from hyaline to yellow, pink, brown and black. The wall may be smooth or show one of many distinct surface textures. The average size is 10 μ though they may be as small as 4 μ (<u>Stemonitis smithii</u>) or as large as 20 μ (<u>Fuglio megaspora</u>). These characters are constant and are useful means of identification.

<u>Swarm cells and myxamoebae</u>. One to four of these arise from the germinating spore. What happens in nature is unknown, but in culture if the spore is germinated in water the protoplasts released are flagellate, if it is germinated on a moist surface devoid of free water they are amoeboid. The amoebae are up to 30 μ in diameter. They show no polarity and in general show similar characteristics to other amoebae e.g. they phagocytose and pinocytose and divide by mitosis and cytokinesis. The swarm cells are now believed to be biflagellate. After germination a 10-15 μ long flagellum develops, followed later by a very much shorter, recurved flagellum [5,6]. The long flagellum has been demonstrated to be constructed on the typical 9 + 2 pattern[5]. Flagellum-like pseudopodia have been seen to originate at the base of the flagellum and migrate toward the posterior end[7]. Streaming of the swarm cell is easily observed, and the stickiness and pseudopodial activity of the posterior end have been

demonstrated. The swarm cell absorbs food from its surround-ing medium[8,9] and ingests bacteria with the pseudopodia at its posterior end.

It has been reported that myxamoebae are produced by the advancing fan of the plasmodium of _Arcyia cisserea_ and _Stemonitis herbatica_[10]. This was later confirmed in a species of _Physarum_[11]. The myxamoebae are cut off, and migrate away from the plasmodium.

Microcysts. In unfavorable conditions such as over-crowding, lack of food, accumulation of various metabolites, drought or overabundance of water, myxamoebae encyst. The conditions which cause encystment have not been studied pre-cisely, but a reversal of the conditions that cause encyst-ment often initiate a resumption of activity. As described for _Fuglio cinerea_ and _Physarum gyrosum_ encystment occurs as follows:

A swarm cell resorbs its flagellum and rounds up. The cytoplasm darkens and the cell volume decreases, indicating a condensation of the cytoplasm. The nucleus occupies a central position and also decreases in volume. Mitochondria accumulate around the nucleus. The contractile vacuole ceases to function and disappears. Food vacuoles disappear. Finally, a thick, refractile membrane forms around the cell[12].

Plasmodia. The simplest view of plasmodium formation is that two haploid myxamoebae or swarm cells, either homo-thallic or heterothallic, fuse. The plasmogamy is followed

by karyogamy to form a diploid zygote which can then develop
into a plasmodium by nuclear divisions without cell divisions.
It has been observed that many zygotes may coalesce, but not
with sufficient frequency to say whether it is accidental or
a result of active aggregation. The existence of apogamy
in Myxomycetes is controversial.

There is little work on the factors affecting plasmodium
formation. Some very early work investigated various physico-
chemical criteria. More recently, ways of inhibiting plasmo-
dium formation have been found; 2% brucine (or glucose)
added to SM/5 medium has inhibited plasmodium formation of
Didymium nigripes. The brucine and glucose may act by prevent-
ing fusion, or, if fusion occurs, by preventing the plasmodia
from enlarging. The latter is less likely because plasmodia
are easily distinguishable from myxamoebae and it is assumed
that they would have been detected if present[13]. The optimal
concentration of myxamoebae for plasmodium formation in a
strain of Didymium nigripes has been stated to be 2.5 x 10^5/ml.[14]
Work has been done on the effect of temperature and light on
plasmodium formation in a species of Physarum. It showed
that light has no effect but an increase in temperature short-
ens the time required for plasmdium formation to a minimum
of 3 days at 25° C(the maximum being 27 days at 15° C).
However the number of cultures forming plasmodia was less
dramatically affected. It is possible that the pronounced
effect of temperature on the time required for plasmodium
formation was also due to a retardation of spore formation[15].

Free water may or may not be necessary for plasmodium formation.

The plasmodium itself is a naked, free living, multi-nucleate, motile mass of protoplasm, usually colorless or some shade of yellow. For studies on plasmodia it has been pointed out that for most purposes the best myxomycete is Physarum polycephalum[16].

There are three types of plasmodium:

1) Protoplasmodium. This is produced by the small myxomycetes such as Echinostelium minutum and Licea parasitica. It has no veins and a very slow, irregular protoplasmic streaming.

2) Aphanoplasmodium. This is produced by Stemonitis fusca and Comatricha laxa, for example. It may live within the woody substratum on which its fruitings are found. However it is flat, transparent and not easily detectable, and may be on the surface. It forms a mesh of hypha-like branches which develop from the original protoplasmodium-like mass. The mesh may be up to 400 μ in diameter. There may be regular reversible protoplasmic streaming. As it gets older one or more fans with definite margins may be formed. It remains very flat and transparent and therefore almost invisible to the naked eye.

3) Phaneroplasmodium. This is the best known of plasmodia and are reported in all species of Physarales studied. They are usually a few cm. square but plasmodia of Fuglio septica have been reported with an area of up to 1 sq. ft.

The development is via a minute plasmodium which shows stream-
ing when it is at a size of 40 μ. The plasmodium elongates
and forms a channel in which the protoplasm begins a rhythmic
reversible streaming. The plasmodium develops into a fan-
shaped structure which shows a distinct differentiation be-
tween an outer, jellified, non-streaming region and an inner,
more fluid, streaming region. The fan thickens and streaming
channels develop. The main channels run from front to back
and are cross connected by smaller channels. The phaeroplasmo-
dium has a three-dimensional appearance.

Phaneroplasmodia can be subcultured by transferring bits
of plasmodium (as small as 0.01 mg) to fresh substrate. The
plasmodium can overcome its injury provided that care is
taken. The plasmodium and propagules survive best if fragments
are taken during the period of optimum growth (8-day-old cul-
tures on oat agar). The good survival of plasmodia after
such operation is due to the rapid gelling of the plasmasol,
and the speed of membrane formation. It has been suggested
that new plasma membrane may be formed from the limiting mem-
branes of the many vesicles[17]. The plasmodium is surrounded
by a sheath, and it is the discarded sheath, left behind by
the plasmodium as it crosses its substrate that is the
"slime track" that gives the Myxomycetes their name. It
has been determined that the sheath has a fibrillar struc-
ture and it is suggested it might be a sort of exoskeleton[18,19].

Plasmodial nuclei of P. polycephalum grown axenically in liquid medium were found to be 4-5 μ in diameter, and contained a single nucleolus[20]. There are a large number of nuclei in the plasmodium. In P. polycephalum there are approximately $8 \times 10^5/mm^3$, which means that 7% of the protoplasm consists of nuclei, as compared with 6% in the amoeba Chaos chaos[21]. The protoplasm of the plasmodium streams, and because the direction of streaming varies rhythmically it has been named shuttle streaming. The rate of flow is very high, 1350 μ/sec. for P. polycephalum[22] as compared with flow rate of 250 μ/sec. in a fungus hypha[23] and 6 μ/sec. in a plant[24]. There are at least thirteen hypotheses to explain the mechanisms of streaming: surface tension, hydration, osmosis, sol-gel reversability, myelin processes, coacervates, autonomous propulsion of particles, kinetic energy, magnetism electrical forces, contractility[25], diffusion drag[26] and contraction-hydraulic[27].

Sclerotia. In unfavorable conditions the plasmodium may become a sclerotium. This is a dry horny irregular mass which is considerably more resistant than the naked plasmodium and may remain viable for up to a year. Sclerotia of P. polycephalum may be produced in the lab by overfeeding plasmodia with rolled oats and drying at between 18°-32° C.[28] The stages of sclerotium formation are as follows:

cessation of streaming

gelation of the whole structure

distribution of nuclei

depositing of wall

completion of macrocyst formation

hardening of the sclerotium

shrinkage of nuclei to half-size.

Sporulation and the Sporophore. The factors responsible
for the cessation of assimiliation and the initiation of
sporulation, although well studied are not completely under-
stood. Studies include the effect of light - continuous
versus intermittent[29], intensity[30], wavelength[31,32], the
effect of u.v.[32,33], the effect of temperature and pH[34], and
genetic control[35]. It is concluded that:

1) Light is necessary for the induction of sporulation
in yellow pigmented plasmodia.

2) Niacin is essential for the induction of light sensi-
tivity.

3) The nutritional history is a very important factor in
determining whether or not sporulation occurs, and the time
of sporulation.

4) the presence of -SH groups prevents a plasmodium from
sporulating.

The shape of the sporophore is species specific and
is the basis of myxomycete classification. There are four
types of sporophore:

1) the sporangium, - sessile or stalked, may have a capillitat system.

2) the plasmoidiocarp - similar to a sessile sporangium but elongated and lying flat on the substrate

3) the aethelium - large, thought to be fused sporangia.

4) the pseudoaethelium - intermediate between sporangiea and aethelia.

The capillatum is a network of threads within the fruiting bodies of many Myxomycetes. These threads are intermingled with the spores but not connected to them since they have been formed by a completely separate developmental system.

4. Culture of Myxomycetes

1. Crude culture. Phaneroplasmodia may be grown on Knops agar or CM/2 agar at 20-35° C. The plasmodium is fed by sprinkling it with sterile pulverized rolled oats. Vigorous plasmodium fans may be cut and transferred to new petri dishes to propagate or maintain the culture. Some Myxomycetes prefer oat flake agar.

2. Spore-to-spore culture. Spores are spread on CM/2 agar and the surface is flooded with sterile distilled water. After about 3-6 days at 20-25° C plasmodia should have formed (check by microscopic examination). When this has occurred, feed with sterile pulverized rolled oats. With some species the transfer of the plasmodium to non-nutrient agar is sufficient to induce sporulation (Fulgio cinerea, Didymium squamulosum, D. iridis, D. nigripes, Physanum pusillum and P. didermoides.) Other Myxomycetes require other factors to induce sporulation, and these have not yet been defined.

3. Monaxenic culture. The procedure is as follows: A spore suspension is made by crushing one or more sporangia in 10 ml sterile distilled water. 3 drops of spore suspension are placed near the edge of a CM/2 agar plate which is tilted, allowing the drops to run across to the other side. The dish is incubated at 25° C and examined periodically for a myx-amoebal population which should migrate away from the bacteria growing along the inoculation streak. With a flamed needle cut out a block of agar bearing several myxamoebae, and free from bacterial colonies as determined by at least X90

microscopical examination. Transfer the block to a petri
dish of sterile CM/2 agar and add 0.5 ml of an E. coli
suspension. When plasmodia form, feed with sterile pulverized
oats. When fruiting occurs new monaxenic should be started
from the spores. To check the purity of the cultures 5 agar
blocks in the vicinity of the first should be cut. One
should be placed in each of the 5 tubes containing

1) Nutrient broth

2) Difco Ac broth

3) Yeast extract broth

4) Thioglycolate broth

5) Glucose broth

These tubes should be incubated at 25° C and examined peri-
odically for turbidity. If any turbidity is found in any
tube within 30 days, it should be assumed that the myxamoebae
isolated were contaminated, and the culture should be dis-
carded. If the tubes remain clear it is safe to assume
that a monaxenic culture has been established.

Axenic Culture. The phaneroplasmodium should be rid of fun-
gus by allowing it to migrate on CM/2 agar and transferring
a piece of fungus-free fan to oat-flake agar. This is very
difficult as often the fungi outgrow the plasmodium. If it
is accomplished the plasmodium should be allowed to grow
until large, vigorous fans are produced (3-7 days). A piece
of fan should then be transferred to a 2% agar plate con-
taining potassium penicillin (2×10^3 units/ml) and

streptomycin (2 mg/ml) and allowed to migrate. Six hours is sufficient. Test for bacterial contamination as described for monoxenic cultures.

Physarum polycephalum has been grown on a semi-synthetic medium containing chick embryo extract[36*]. Subsequent analysis of the organism's requirement for chick embryo extract showed that this could be replaced by haematin or certain haemato-proteins[37,38]. The partially defined medium for P. polycephalum could be replaced by 3 completely defined synthetic media which gave cell yields of approximately 70% of those obtained in a partially defined medium[39]. Some modifications were made so that the plasmodia of Physanum flavicomum and Physarella oblonga and the myxamoebae of Badhamia curtisii could be grown in this medium[40]. The medium was further modified to allow the growth of plasmodia of Physarum rigidum[41].

Myxamoebae of Didimium nigirpes have been grown in the presence of bacteria killed with formalin and also bacteria disintegrated by treatment with ultrasonics or a Waring blender and filter sterilized[42]. Didymium nigripes and Physarum cinereum have also been grown in the presence of heat-killed bacteria[43].

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* No. 9 in appendix

5. Maintainance and Storage

Myxamoebae. These can be stored on CM/2 agar slants with a few ml bacterial suspension at 10-15° C. With occasional transfers such clones have remained viable for several years[15]. For longer storage lyophilization or liquid nitrogen storage is recommended[44]. Myxamoebae of Didymium nigripes have been maintained (i.e. prevented from forming plasmodia) by growing them on SM/5 agar with a 48 hour culture of Aerobacter aerogenes and the addition of 2% glucose or brucine to the medium. The myxamoebae survived and continued to multiply until the plate was cleared of bacteria. On subculture to similar media this process was repeated[13].

Plasmodia. Monoxenic or axenic cultures may be maintained for many years on oatmeal agar in the dark at 10-15° C. They should be transferred once a month to keep them in vigorous condition. They can also be kept as sclerotia for one or two years at 10-15° C and placed on moist agar to reconstitute the plasmodia.

Spores. These should be stored dry in the sporangia in which they were formed. If collected in the field they should be stored with an insecticide (e.g. PDB (paradichlorobenzene) to protect from destruction by insects. Spores do not usually survive for more than 3 or 4 years. If the spores are lyophilized they may survive for more than 5 years[45].

6. Media

1) CM/2 Agar:

	Cornmeal Agar	8.5 g
	Bacto Agar	12.5 g
	Distilled water	1000.0 ml

2) Knop's solution Agar:

	$Ca(NO_3)_2 \cdot 4H_2O$	0.8 g
	KNO_3	0.2 g
	KH_2PO_4	0.2 g
	$MgSO_4 \cdot 7H_2O$	0.2 g
	$FeSO_4$	"a trace"
	Bacto Agar	20.0 g
	Distilled water	1000.0 ml

3) Oat Flake Agar: Place enough oat flakes (rolled oats) preferably of the slow-cooking type to form a 5 mm layer in 100 x 20 mm Petri dishes. Pour enough hot melted Bacto agar in each dish to just cover the oats. Autoclave for 1 hour + at 115 lb/sq. in.

4) Nutrient broth:

	Bacto nutrient broth	8.0 g
	Distilled water	1000.0 ml

5) AC broth:

	Bacto-AC medium	34.0 g
	Distilled water	1000.0 ml

6) Yeast-extract broth:

	Yeast extract	1.0 g
	Distilled water	1000.0 ml

7) Thioglycolate broth:

	Bacto-thioglycolate medium	24.0 g
	Distilled water	1000.0 ml

8) Glucose broth:

	Glucose	1.0 g
	Bacto peptone	1.0 g
	Distilled water	1000.0 ml

9) Daniel & Rusch's semi-synthetic medium:[36]

tryptone	1.0 g
yeast extract	0.15 g
glucose	1.0 g
KH_2PO_4	0.2 g
$CaCl_2 \cdot 2H_2O$	0.06 g
$MgSO_4 \cdot 7H_2O$	0.06 g
$FeCl_2 \cdot 4H_2O$	0.006 g
$MnCl_2 \cdot 4H_2O$	0.0086 g
$ZnSO_4 \cdot 7H_2O$	0.0036 g
citric acid $\cdot H_2O$	0.048 g
HCl conc.	0.006 g
$CaCO_3$	0.3 g
chick embryo extract	1.5 ml
distilled water to	100 ml

10) SM/5 Medium:[13]

Bacto peptone	2.0 g
Glucose	2.0 g
Yeast extract	0.2 g
$K_2H PO_4$	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Bacto agar	20.0 g
Distilled water	1000.0 ml

7.   Some Recent Studies on Myxomycetes

1)   Genetic: Plasmodia may or may not fuse depending on wheth-
er they have genetic similarities in certain loci. This
phenomenon has been studied and two explanations have been
proposed. For Didymium iridis a polygenic system with simple
dominance at each locus has been postulated[46, 48]. For
Physarum polycephalum there is some disagreement; either the
system is polygenic as in D. iridis[49], or it is a one locus,
multiple allelic system[59,51].

2)   Biochemical: The plasmodium of Physarum polycephalum
has been used for the study of the effects of dithiocarbamates
with the result that respiration is strongly inhibited at
285 mg/1[52]. It was also found to be effective as a screen for
95 out of 144 antitumor agents tested at a concentration of
less than 100 μg/ml.[53] Synthesis of DNA in the plasmodium
occurs immediately after telophase (there is no G1 phase),
and simultaneously in 99[+]% of the nuclei[54,56]. Mitosis can be
induced in nuclei as soon as DNA synthesis has been completed,
as has been demonstrated by fusing small plasmodia, whose nuclei
have just completed DNA synthesis, with a large plasmodium whose
nuclei are in prophase. When the nuclei from the large plasmodium
undergo mitosis, the nuclei from the small plasmodium do so, too[57].
This result, plus the fact that it appears unlikely that a high
degree of mitotic synchrony would be able to occur if mi-
tosis was governed by nuclear stimulation, suggests that
the trigger for nuclear division resides in the cytoplasm[58].
Further biochemical events in the nuclear cycle are documented.

in greater detail elsewhere[59]. Ribonucleases and nucleases
have been isolated from P. polycephalum and studied[60-62].
The reasons given for choosing a myxomycete were that no myxo-
mycete ribonuclease had previously been studied, that the
knowledge of the properties of such an enzyme would be useful
when characterising Physarum RNA and as Physarum is highly
synchronous with respect to nuclear fussion it is suitable
for studies of the biochemistry of the cell cycle. Other
enzymes studied in Physarum are a protein kinase which was
found to be inhibited by cyclic AMP[63]; a glutamate decar-
boxylase (g.d.) which was found to be similar in some respects
to g.d. of mouse brain but an optimum pH nearer to the opti-
mum pH of g.d. of higher plants, and a thermal stability
greater than that reported for the g.d. of any other
organism[64]. Isocitrate dehydrogenase glucose-6-phosphate
dehydrogenase, glutamate dehydrogenase, acid phosphatase,
phosphodiesterase, β-glucosidase and histidase were studied
in growing plasmodia of P. polycephalum. 6 of the enzymes
showed a continuous increase in activity during the mitotic
cycle, showing that the mRNA for all 7 enzymes is stable
during the G2 phase[65].

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